Construction and Characterization of *Escherichia coli* O157:H7 Strains Expressing Firefly Luciferase and Green Fluorescent Protein and Their Use in Survival Studies‡

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(MS# 96-194: Received 31 July 1996/Accepted 17 January 1997)

ABSTRACT

The firefly (*Photinus pyralis*) luciferase (*luc*) gene on plasmid vector pBESTluc and the *Aequorea victoria* green fluorescent protein (*gfp*) gene on plasmid vector pGFP were introduced into strains of *Escherichia coli* O157:H7. The recombinant *E. coli* strains were indistinguishable from their parent strains in biochemical and immunological assays and in a multiplex PCR reaction. There was no significant difference in the growth kinetics of the *luc*-bearing recombinants and the parent strains. At 37°C all of the recombinant strains maintained the vectors and expressed luciferase and the green fluorescent protein when grown both with and without antibiotic selection. Individual colonies of *luc*-bearing *E. coli* strains were readily luminescent in the dark after being sprayed with a solution of 1 mM beetle luciferin. The recombinants containing pGFP emitted bright green fluorescence when excited with UV light and the addition of any other proteins, substrates, or cofactors was not required. The green fluorescent protein-expressing *E. coli* O157:H7 strains were used in studies examining the survival of the organism in apple cider and in orange juice. In apple cider the organism declined to undetectable levels in 24 days at refrigeration temperature while in orange juice the strains survived with only small decreases in number during the 24-day sampling period. These recombinant *E. coli* O157:H7 strains, containing readily identifiable and stable markers, could be useful as positive controls in microbial assays as well as in studies monitoring bacterial survival and the behavior of *E. coli* O157:H7 in foods and in a food processing environment.

Key words: Firefly luciferase, green fluorescent protein, *Escherichia coli* O157:H7

Enterohemorrhagic *Escherichia coli* O157:H7 is now recognized as an important etiologic agent of hemorrhagic colitis and a leading cause of hemolytic uremic syndrome *(8, 11, 21)*. The pathogen has been associated with numerous outbreaks and sporadic cases of disease, epidemiologically linked most frequently to foods of bovine origin *(8, 11, 21)*. Due to the increasing public health significance of *E. coli* O157:H7, much attention has focused on the development of rapid methods for detection of the organism in foods *(19)*. The efficacy and accuracy of laboratory procedures for the isolation and detection of various bacteria from food samples must be evaluated regularly. Specifically, *E. coli* O157:H7 positive controls possessing appropriate markers are needed to distinguish control strains from strains of *E. coli* O157:H7 isolated from food samples. *E. coli* O157:H7 strains, potentially strains with reduced virulence and containing, e.g., luminescence markers, can be utilized as laboratory positive control strains and also to monitor microbial cross-contamination in food processing plants *(17)*, to monitor microbial growth, survival, and colonization under various conditions *(4, 9, 10, 23, 27)*, and to test the effects of antibiotics, chemicals, and preservatives on the bacteria *(15, 18)*.

The luciferase enzyme, isolated from the North American firefly (*Photinus pyralis*), has been extensively characterized; this enzyme has been inserted into bacteria where it can catalyze the production of light *(7, 28)*. The reaction involves the oxidation of the substrate D-luciferin in the presence of ATP resulting in the production of oxyluciferin, AMP, PP_i, and light. The green fluorescent protein, produced by the jellyfish *Aequorea victoria*, is currently attracting much interest, and the utility of this protein for various applications has been described *(3)*. In the jellyfish, upon transfer of energy from a Ca^{2+}-activated photoprotein, aequorin, to the green fluorescent protein, green fluorescent light is emitted *(13)*. The *gfp* gene has been cloned *(22)* and expressed in heterologous systems *(1, 13)*, both eukaryotic and prokaryotic. The 27-kDa green fluorescent protein (238 amino acid residues) encoded by the *Aequorea gfp* gene absorbs UV and blue light with a peak of maximum absorbance at 395 nm and emits green light at 509 nm. The protein is highly stable to heat, alkaline pH, detergents, and many proteases *(3, 26)*, and fluorescence is not dependent on the presence of any other proteins, substrates, or cofactors

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(14). This report describes the construction and characterization of bioluminescent *E. coli* O157:H7 strains designed for use as positive controls in microbial assays and also the utilization of the recombinant strains in survival studies in unpasteurized apple cider and orange juice.

MATERIALS AND METHODS

Bacterial strains

The following *E. coli* O157:H7 strains were used in this study: 43894 (American Type Culture Collection, Rockville, MD), B6-914 (Centers for Disease Control and Prevention, Atlanta, GA), and 380-94 (Food Safety and Inspection Service, Washington, D.C.). Strain B6-914 does not produce Shiga-like toxins I or II (also referred to as Stx1 and Stx2, respectively) and, therefore, may be less hazardous to laboratory personnel. These three strains were maintained on Luria-Bertani (LB) agar *(24)* and for use were grown in LB broth.

Plasmids

The plasmid vector pBESTluc, containing an ampicillin resistance gene and the eukaryotic firefly luciferase (*luc*) gene positioned downstream from the *tac* promoter and a ribosome binding site, was obtained from Promega Corporation, Madison, WI (4,486 bp; *E. coli* S30 extract system for circular DNA kit). The pGFP cDNA vector (3,344 bp; Clontech Laboratories, Inc., Palo Alto, CA) contains the complete green fluorescent protein coding sequence inserted in frame with the *lacZ* initiation codon from pUC19, a high-copy-number origin of replication, and an ampicillin resistance gene.

Construction and identification of bioluminescent E. coli *strains*

Competent cells of *E. coli* O157:H7 strains were prepared and the plasmids pBESTluc and pGFP were introduced into the competent *E. coli* by the calcium chloride method as described by Sambrook et al. (Protocol II) *(24)*. Transformants were selected by growth on LB agar containing 100 μg/ml of ampicillin and on MacConkey sorbitol agar containing ampicillin (100 μg/ml) and supplemented with 5-bromo-4-chloro-3-indoxyl β-D-glucuronide (MSA-BCIG) *(20)*. Bioluminescent transformants containing pBESTluc were identified by visual inspection of colonies in the dark. Colonies were luminescent only after being sprayed with 1 mM beetle luciferin (Promega) prepared in 0.1 M sodium citrate, pH 5.5. To examine plates for colonies formed by transformants containing pGFP and expressing the green fluorescent protein, the plates were illuminated with an ultraviolet lamp (366 nm, Mineralight Lamp, Ultra-Violet Products, Inc., San Gabriel, CA) held directly over the opened plate at a distance of approximately 4 to 6 inches. Fluorescent colonies were immediately visible.

Transformants containing pGFP and the parent strains were grown at 37°C on glass slides coated with a thin layer of SOB agar *(24)* containing 100 μg/ml of ampicillin and 1 mM isopropylthio-β-galactoside (IPTG). The SOB agar on which the parent strains were grown did not contain ampicillin or IPTG. Colonies were examined using a Nikon Diaphot-TMD microscope (Nikon, Inc., Melville, NY) equipped with a B-2A filter cube and photographed using TMAX ASA 400 film. Exposure times and control of a 35-mm camera were through a Nikon UFX-DX photographic attachment.

Biochemical tests and immunoassays

The *E. coli* strains were tested using the API 20E System (bioMérieuxVitek, Hazelwood, MO) according to the manufacturer's instructions. Three immunoassays were used in this study: the coline *E. coli* O157:H7 visual line immunoassay (Integrated BioSolutions, Inc., Monmouth Junction, NJ); RIM *E. coli* O157:H7 (Remel, Lenexa, KS), a latex test for presumptive identification of *E. coli* serogroup O157 cultured on laboratory media; and a direct immunofluorescence technique using fluorescein isothiocyanate (FITC)-labeled antibody against *E. coli* O157 (0.5 mg/ml, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). In the use of the coline and RIM *E. coli* O157:H7 assays, the manufacturers' instructions were followed. With the immunofluorescence technique, a loopful of a bacterial colony was suspended in 50 μl of sterile distilled water (SDW) in a sterile 1.5-ml centrifuge tube and mixed with 5 μl of the FITC-labeled antibody against *E. coli* O157. The mixture was incubated without shaking for 30 min in the dark at room temperature. Following centrifugation for 2 min at 16,000 × *g*, the pellet was suspended in 500 μl of SDW, centrifuged as above, and resuspended in 50 μl of SDW. A 10-μl portion of the bacterial suspension was placed on a glass slide and examined by epifluorescence microscopy for the presence of bright yellow-green fluorescent bacteria.

Polymerase chain reaction (PCR)

PCR was performed using *E. coli* multiplex PCR tablets as described previously *(6)*. Briefly, colonies were individually suspended in 50-μl volumes of SDW in MicroAmp PCR reaction tubes (Perkin Elmer Cetus, Norwalk, CT) and heated at 99°C for 10 min using a Model 9600 DNA thermal cycler (Perkin Elmer Cetus) to lyse the bacteria and denature the DNA. For DNA amplification, 5-μl volumes of target DNA, 10 μl of 5× PCR buffer, pH 8.0 (50 mM Tris-HCl, 250 mM KCl, 7.5 mM $MgCl_2$, and 0.5% Triton X-100), and a PCR tablet were added to each tube. The reaction mixtures were heated at 94°C for 2 min and then subjected to 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min with an additional 10-min extension at 72°C. The PCR was designed to simultaneously amplify different DNA sequences of *E. coli* O157:H7 in a single reaction resulting in three amplification products: a specific fragment of an attaching and effacing (*eaeA*) gene (670 bp), conserved sequences of Shiga-like toxin (SLT) I and II (305 bp) genes, and a fragment of the 60-MDa plasmid (166 bp). PCR was also performed to confirm the presence of the luciferase and green fluorescent protein coding sequences in the transformants. Primers for the PCR were designed using the OLIGO primer analysis software (National Biosciences, Inc., Plymouth, MN) and prepared by the University of Kentucky, Macromolecular Structure Analysis Facility, Lexington, KY. The primer sets for amplification of the *luc* gene and *gfp* cDNA were, respectively, 5′-ATAGAACTGCCTGCGTCAGA-3′ (lucf, forward), 5′-CCCTCGGGTGTAATCAGAAT-3′ (lucr, reverse) (430 bp product), and 5′-GAAGATGGAAGCGTTCAACT-3′ (gfpf, forward), 5′-TTCACCGTCATCACCGAAAC-3′ (gfpr, reverse) (330 bp product). One colony of each of the recombinant and parent strains was suspended in 50 μl of SDW and the samples were heated at 99°C for 10 min. The PCR reaction (50 μl) consisted of 5 μl of target DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) of the 4 deoxynucleoside triphosphates (Gibco/BRL Life Technologies, Inc., Gaithersburg, MD), 2.5 U of *Taq* DNA polymerase (Gibco/BRL), and 50 pmol of each of the two primers. The reaction mixtures were heated at 94°C for 1 min, then subjected to 35 cycles of denaturation at 94°C for 45 s and annealing and extension for 1 min at 68°C, with an additional 5-min extension at 68°C. The reaction mixtures were subjected to agarose (1.5%) gel electrophoresis and gels were stained with ethidium bromide.

Growth kinetics

Cultures of both parent and *luc* recombinant strains were inoculated into 50-ml portions of LB broth in 250-ml Erlenmeyer flasks for a final concentration of 100 CFU/ml. Uninoculated LB broth was used as a negative control. All flasks were incubated at 37°C on a rotary shaker (100 rpm). A 2-ml sample was removed from each flask at hours 0, 1, 2, 4, 6, 8, 12, and 24, plated onto nutrient agar (NA; Difco, Detroit, MI) using a Spiral Plater (Model D, Spiral Biotech, Inc., Bethesda, MD), and following overnight incubation of the plates at 37°C colonies were enumerated using a Laser Counter (Spiral Biotech). The overall growth rates (CFU/ml/h) of the bacterial strains were compared.

Determination of stability of bioluminescent E. coli *strains*

Liquid cultures (10 ml of LB broth) of the recombinant *E. coli* strains were grown overnight with and without antibiotic (ampicillin) selection at both 37 and 42°C. Ten-microliter portions of the overnight cultures were then used to inoculate fresh LB broth to be incubated at the same temperatures for 24 h both with and without antibiotic selection. Cultures grown with ampicillin were passaged into LB medium containing the antibiotic and cultures grown without the antibiotic were passaged into LB lacking ampicillin. To examine for the presence of the *luc* gene or *gfp* cDNA-containing plasmids in *E. coli* cells, following each passage at each temperature, a 3-mm loopful of each of the cultures was plated on LB agar containing 100 µg/ml ampicillin and incubated for 24 h at the same temperature under which the passage in LB medium was performed. Bacterial colonies on the agar plate were examined for bioluminescence as described above. This process was repeated for 15 days, or until no colonies were detected on selective agar plates.

Survival in apple cider and orange juice

Fresh unpasteurized apple cider was purchased from a local producer in Doylestown, PA, and used 3 days following storage at 4°C. Unpasteurized fresh-squeezed orange juice was purchased from a local producer in Elkins Park, PA, and used following storage at 4°C for 24 h. Overnight cultures of strains gfp-1 and gfp-29, grown in tryptic soy broth (Difco), were inoculated into the cider and juice (50 ml) at a level of approximately 1×10^4 CFU/ml. Inoculated and uninoculated cider and juice were kept at 4°C and periodically samples were removed, diluted, and plated onto tryptic soy agar (TSA, Difco), NA, and APT agar (Difco). The plates were incubated at 37°C for 18 h. All colonies which formed from the uninoculated control samples were hand counted. With the inoculated samples, the fluorescent *E. coli* O157:H7 colonies were visualized using an ultraviolet lamp as described above and were counted. The pH in cider and juice was measured several times during the 24-day sampling period.

RESULTS

Construction of bioluminescent strains of E. coli *O157:H7*

The *luc* gene and *gfp* cDNA-containing plasmid vectors, pBESTluc and pGFP, respectively, were successfully introduced into the three strains of *E. coli* O157:H7: 43894, B6-914, and 380-94. The recombinant *E. coli* strains containing pBESTluc were designated as luc-1 (derived from 43894), luc-2 (B6-914), and luc-3 (380-94) and those containing pGFP were designated gfp-1 (43894), gfp-29 (B6-914), and gfp-56 (380-94). Colonies of the recombinant and parent strains had identical morphological characteristics on LB agar. Colonies of *luc* recombinant strains became luminescent in the dark after being sprayed with 1 mM beetle luciferin (Fig. 1) and colonies of recombinant strains containing pGFP produced green fluorescence when exposed to light at 395 nm (Fig. 2).

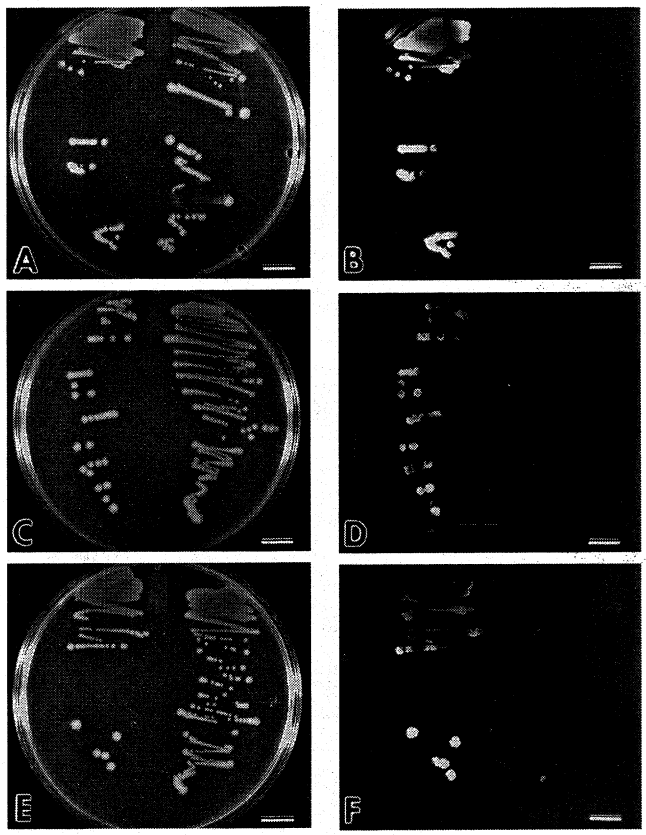

FIGURE 1. *Visualization of colonies of* E. coli *O157:H7 strains on LB agar plates in the light (A, C, and E) and in the dark (B, D, and F). The agar plate in A was streaked with bioluminescent* E. coli *O157:H7 strain luc-1 (left half of the plate) and its parent strain 43894 (right half), incubated at 37°C for 24 h and photographed in the light. All the colonies on the agar were then sprayed with 1 mM beetle luciferin prepared in 0.1 M sodium citrate, pH 5.5, inspected and photographed in the dark. Photographs for C and D and E and F were obtained in the same manner as for A and B. In C, bioluminescent strain luc-2 was plated on the left half of the plate and the parent strain B6-914 was on the right half. In E, luc-3 was on the left and strain 380-94 was on the right.*

Biochemical tests and immunoassays

Using the API 20E system, the recombinant bioluminescent strains and their parent strains 43894, B6-914, and 380-94 had similar biochemical reactions typical of *E. coli* O157:H7. No differences were observed with the three immunoassays used in this study, namely the coline immunoassay, the latex agglutination test, and the direct immunofluorescence assay. The recombinant and parent strains were identified as *E. coli* O157:H7.

PCR

All recombinant and parental strains of *E. coli* O157:H7 were tested by PCR using *E. coli* O157:H7 multiplex PCR tablets. There was no difference in PCR results between the recombinant and their parent strains. As shown in Figure 3, amplification products of the expected sizes for *eaeA*, SLT,

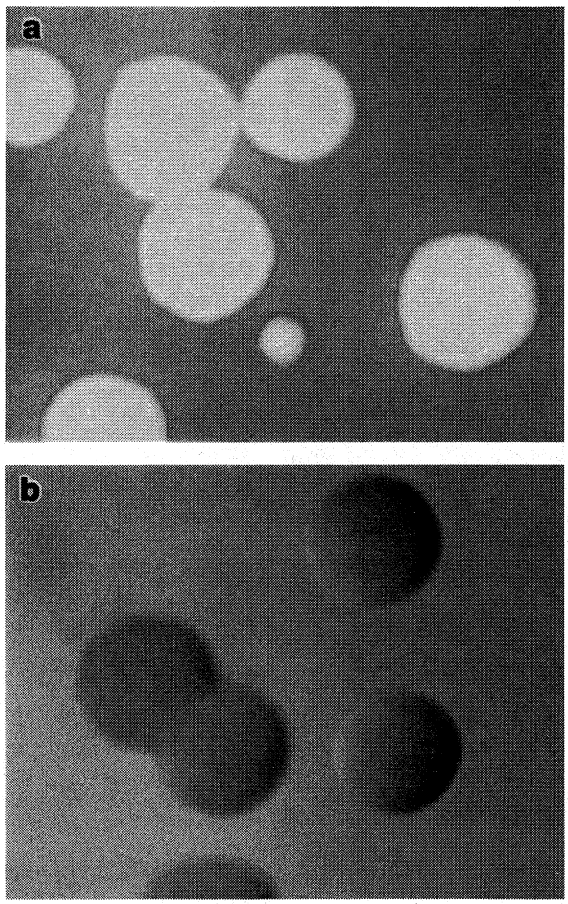

FIGURE 2. *Colonies of* E. coli *O157:H7 ATCC 43894 recombinant (gfp-1) (a) and parent (b) strains examined using a Nikon Diaphot-TMD microscope equipped with a B-2A filter cube. Similar results were obtained with gfp-29 and parent strain B6-914 and with gfp-56 and parent strain 380-94.*

and the 60-MDa plasmid gene fragments were obtained with strain luc-1 (lane a) and its parent strain 43894 (lane b) as well as with strain luc-3 (lane e) and its parent strain 380-94 (lane f). As expected, luc-2 (lane c) and its SLT-negative

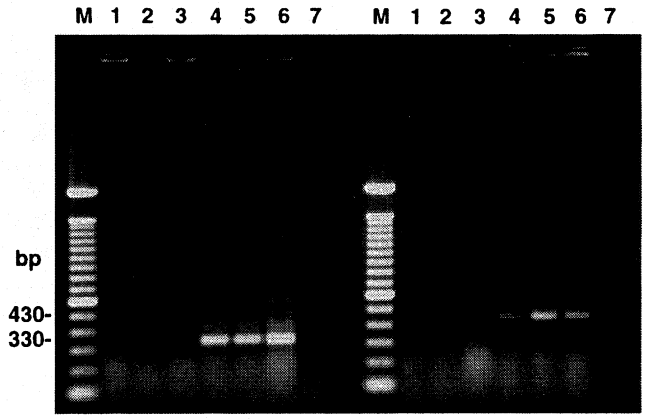

FIGURE 4. *Agarose gel analysis with ethidium bromide staining of PCR products obtained from recombinant and parent strains. The left-hand portion of the gel shows PCR products (330 bp) obtained using primers for amplification of the* gfp *gene; lanes 1–7:* E. coli *43894, B6-914, 380-94, gfp-1, gfp-29, gfp-56, and* H_2O *negative control, respectively. The right-hand portion shows products (430 bp) obtained using primers for amplification of the* luc *gene; lanes 1–7:* E. coli *43894, B6-914, 380-94, luc-1, luc-2, luc-3, and negative control, respectively. Lane M, DNA size markers (1-kb ladder; Gibco/BRL).*

parent strain B6-914 (lane d) showed only amplification products of the *eaeA* gene and the 60-MDa plasmid sequence. Recombinant strains containing pGFP gave similar results (data not shown). PCR results of recombinant and parent strains using primers for *luc* and *gfp* cDNA are shown in Figure 4. The bacteria containing pBESTluc showed a PCR product of 430 bp (lanes 4, 5, and 6, right hand side of gel), and those containing pGFP showed an amplification product of 330 bp (lanes 4, 5, and 6, left hand side) as expected, whereas no products resulted following PCR of the parent strains (lanes 1, 2, and 3).

Growth kinetics of recombinant and parent E. coli *strains*

There was no notable difference between the growth kinetics of the bioluminescent *E. coli* strains and the parent

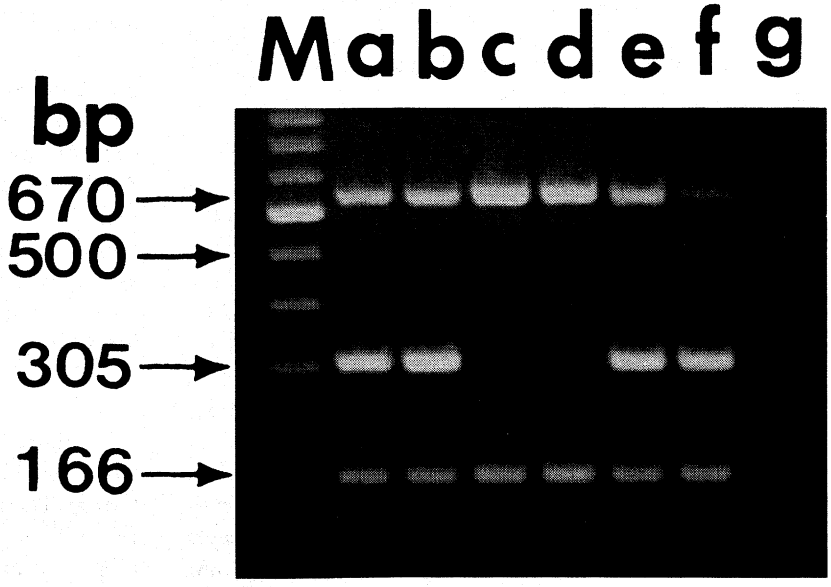

FIGURE 3. *Agarose gel analysis with ethidium bromide staining of multiplex PCR amplification products obtained from strains of* E. coli *O157: H7. Lanes: M, DNA size markers (1-kb ladder; Gibco/BRL); a, luc-1; b, strain 43894; c, luc-2; d, strain B6-914; e, luc-3; f, strain 380-94; g, negative control. Expected sizes of the PCR products of the* eaeA *and SLT genes and 60-MDa plasmid sequence were 670, 305, and 166 bp, respectively.*

strains and no notable difference among the bioluminescent strains. The overall growth rates (CFU/ml/h) during the 24-h incubation period for luc-1, luc-2, luc-3, 43894, B6-914, and 380-94 were 0.355, 0.356, 0.320, 0.354, 0.348, and 0.341, respectively.

Stability and characteristics of bioluminescent E. coli *strains*

Plasmids pBESTluc and pGFP were stably maintained in the recombinant strains through 15 cycles of daily subculturing at 37°C both with and without antibiotic selection. At 42°C, the 6 strains were stable through the 15 cycles of daily passage under antibiotic selection. Without antibiotic selection at 42°C, pGFP was stably maintained, whereas luc-1 and luc-2 lost the *luc*-containing plasmid at passage 15 and luc-3 lost the plasmid at passage 9. With both *luc* and *gfp* recombinant strains, light production and fluorescence, respectively, were strongest when bacteria were grown at lower temperatures, i.e., at 37°C or lower. When IPTG was added to the medium, in general, fluorescence produced by *gfp* recombinant strains appeared somewhat stronger than when using LB without IPTG. In many cases during daily passage, the presence of ampicillin in LB was necessary for strong fluorescent light emission by colonies grown at 42°C. Also, if the plates were reexamined after leaving them at room temperature for several hours, colonies generally appeared more fluorescent. Freezing the recombinant strains at −70°C in 20% glycerol did not affect expression of luciferase and green fluorescent protein when the bacteria were subsequently cultured.

Survival of gfp-1 and gfp-29 in apple cider and orange juice

There was not a notable difference in pH of the apple cider and orange juice samples during the sampling period with pH ranging from 3.35 (day 1) to 3.46 (day 24) in the cider and from 3.51 (day 1) to 3.59 (day 24) in the orange juice. Fluorescence of the *E. coli* O157:H7 colonies appeared very strong on TSA and somewhat less intense on APT agar and NA. In Figure 5, results obtained using TSA are shown. In orange juice, gfp-1 and gfp-29 decreased by 1.48 and 1.84 log cycles, respectively, by day 24. By day 18, populations of gfp-1 and gfp-29 decreased by 1.01 and 1.71 log cycles, respectively, in apple cider; however, by day 24, both gfp-1 and gfp-29 had reached nondetectable levels (<1 CFU/ml). Apparently, *E. coli* O157:H7 is capable of surviving the acidic environment of orange juice longer than that of apple cider. Results were similar using NA and APT. Using TSA, the level of background organisms in the cider and juice increased by 1.43 and 1.84 log cycles, respectively, by day 24.

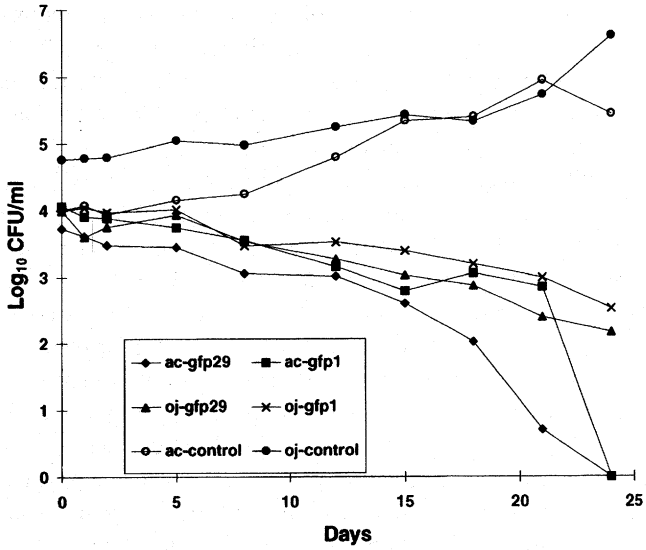

FIGURE 5. *Survival of green fluorescent protein gene-expressing* E. coli *O157:H7 strains (gfp-1 and gfp-29) in unpasteurized apple cider and orange juice incubated at 4°C for 24 days. Samples were plated onto TSA and the fluorescent* E. coli *O157:H7 colonies were counted. The ac-gfp1 and ac-gfp29 curves represent inoculated apple cider samples, and the oj-gfp1 and oj-gfp29 curves represent inoculated orange juice samples. The ac- and oj-control curves represent uninoculated apple cider and orange juice samples, respectively, in which only background organisms were counted.*

DISCUSSION

In the present study, *E. coli* O157:H7 strains containing readily identifiable and stable markers were constructed and characterized. The introduced plasmids conferred resistance to 100 μg/ml of ampicillin and, under this selection, were maintained through at least 15 cycles of daily passage. The pBESTluc- and pGFP-bearing *E. coli* strains were indistinguishable from the parent strains in biochemical and immunological tests and in the multiplex PCR. The assays for expression of firefly luciferase and green fluorescent protein in *E. coli* O157:H7 strains grown on agar plates are quite simple. Recombinant strains expressing luciferase require the addition of substrate directly on the colonies, and light is emitted within seconds, whereas only irradiation by near UV or blue light is required for emission of fluorescence in green fluorescent protein-expressing strains. Because the *luc* and *gfp* genes are not indigenous to *E. coli*, there is no host background.

Cook et al. *(2)* introduced bioluminescence (*lux*) genes from *Vibrio fischeri* and *V. harveyi* into *Bacillus subtilis* on a plasmid vector and found that the maximum specific growth rate of the plasmid-bearing strain was significantly lower than that of its parent strain in LB broth. These authors believed that the reduction in the maximum specific growth rate was most probably due to the metabolic burden imposed on the cell by the maintenance and expression of the *lux*-bearing plasmid. In the present study, no significant differences were observed between the growth kinetics of the bioluminescent *E. coli* O157:H7 strains and of the parent strains when LB broth was used.

Light emission and fluorescence were strongest in the recombinant strains when they were grown at 37°C or below. Other investigators have reported that formation of the green fluorescent protein chromophore is temperature sensitive with *gfp* recombinant *E. coli* showing stronger fluorescence when the bacteria were grown at 24°C *(12)*. In yeast cells expressing the green fluorescent protein, optimum fluorescence was observed in cells grown at 15°C

while cells cultured at 37°C displayed virtually no fluorescence *(16)*. However, when yeast cells were cultured at 23°C then shifted to 35°C, fluorescence was retained. Therefore, once formed, the fluorophore is stable even if the cells are shifted to higher growth temperatures.

Fluorescence also appeared somewhat enhanced when the *gfp* recombinant strains were grown in the presence of IPTG and when the colonies were left at room temperature for several hours after overnight growth at 37 or 42°C. Also, variability in the intensity of fluorescence was often noted among the three recombinant strains. Some of these observations may be due in part to the relatively slow formation of the fluorophore. Heim et al. *(12)* reported that final fluorophore formation in *E. coli* required at least one step with a time constant of 4 h and also required molecular oxygen. Researchers have generated *gfp* mutants with altered fluorophore spectra and improved fluorescence properties *(3, 5, 12)*, and recombinant strains containing these mutated genes may find broader, more innovative applications.

The luminescent recombinant strains of *E. coli* O157:H7 constructed in this study possess easily detectable phenotypic markers. Plasmid pGFP was stable in the bacteria in apple cider and orange juice, and colonies that formed on TSA consistently showed intense fluorescence throughout the 24-day sampling period. Zhao et al. *(29)* found that *E. coli* O157:H7 inoculated at a level of 10^5 CFU/ml survived in different lots of apple cider for 10 to 31 days at 8°C and for 2 to 3 days when the cider was kept at 25°C. In the present study, using the green fluorescent protein-expressing strains at an inoculum level of 10^4 CFU/ml, bacteria survived for at least 21 days in the cider at refrigeration temperatures. Use of selective agars in bacterial survival studies may give lower recoveries due to inability of selective agars to allow recovery of sublethally injured bacteria *(25)*. Using bacteria containing easily identifiable markers allows the use of nonselective agars thus permitting recovery of injured bacteria, and the level of background organisms can also be determined. The recombinant *E. coli* O157:H7 strains should be useful as positive controls for microbial assays, for monitoring the behavior of bacteria in a food processing environment, and in studies examining bacterial injury and recovery and the effects of disinfectants, drugs, and other antibacterial agents on *E. coli* O157:H7.

ACKNOWLEDGMENTS

We thank Dan Solaiman and Eugene Weinberg for critical review of the manuscript, Patricia Klein for assistance in PCR primer design, and Augustine Kim for helpful discussions.

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